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# Pilot Investigation into the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™) for Dengue Detection in East Timor

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## ABSTRACT

Polymerase chain reaction (PCR) assays for detection of vector transmitted virus infections in humans designed for conventional PCR thermal cyclers were modified for real-time PCR using a LightCycler. Two reporter fluorochromes, the double stranded DNA specific dye SYBR Green I, and 6-hydroxy fluorescein conjugated to TaqMan® DNA probes, were employed to detect positive reactions. Following optimisation of the assays for real-time PCR, portable real-time PCR equipment, the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™), was deployed to East Timor and a field trial was conducted from November 2001 until January 2002. Blood samples from a total of 143 army personnel were screened with the R.A.P.I.D.™ for virus infections. These included 33 samples from patients showing clinical symptoms. Results obtained with the R.A.P.I.D.™ were compared to results from serological testing and cell culture analysis.

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# Pilot Investigation into the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™) for Dengue Detection in East Timor

## Executive Summary

Vector transmitted viral diseases pose a considerable health threat to members of the Australian Defence Force (ADF) serving overseas. Dengue fever is of particular concern as indicated by the number of infections reported from service personnel stationed in East Timor. Throughout the Asia-Pacific region, the number of Dengue cases is increasing at a rate that gives reason for concern.

The clinical diagnosis of Dengue fever can be problematic as the acute symptoms of the disease are similar to those caused by hepatitis A, leptospirosis, malaria, typhoid, rickettsial infection and bacterial sepsis. Particularly in South East Asia, this problem is compounded by various vector transmitted viruses that cause Dengue like illness and have to be considered in differential diagnosis. Current clinical diagnosis based on antibodies such as enzyme linked immunosorbent assays (ELISAs) may not provide a definitive result before the fifth or sixth day after the onset of symptoms. Traditional methods based on cell culture isolation and indirect immunofluorescence assays for the detection of viruses require cell culture facilities and take up to seven days for a result.

The polymerase chain reaction (PCR) is an alternative to established procedures of virus detection and identification. The method provides a high level of sensitivity. PCR can detect a single gene of a given target organism and is typically completed within two to three hours, including sample preparation and analysis of PCR products via gel electrophoresis. The method was significantly enhanced with the recent development of real-time PCR utilising reporter fluorochromes that enable monitoring of the reaction during amplification and reduces reaction times to approximately 20 minutes.

Published PCR protocols for detection of viruses were evaluated and subsequently modified and optimised for real-time PCR. A portable thermal cycler for real-time PCR, the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™), was deployed to East Timor, and the modified protocols were applied to screening of clinical specimens from Army personnel over a period of two months. The R.A.P.I.D.™ performed successfully under laboratory and field conditions and proved to be a deployable diagnostic tool with a wide range of potential applications within the ADF.

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# Contents

# 1. Introduction

Over recent years, a significant increase in infections from viruses that are transmitted to humans by the bite of an infected arthropod such as a mosquito, tick or sand fly has been observed in the Asia-Pacific region. These viruses are commonly known as arthropod borne (arbo) viruses. Arboviruses causing disease in humans belong to two groups in current viral systematics, Alphaviruses and Flaviviruses. Relevant species include Chikungunya, Ross River and Barmah Forest viruses (Alphaviruses), and Dengue, Japanese Encephalitis and Murray Valley Encephalitis viruses (Flaviviruses), with Dengue virus infections now recognised as one of the most important public health problems in tropical countries (Gubler 2002). Numerous incidences of Dengue virus infections in military forces have been reported. A major regional pandemic of Dengue serotype 1 occurred in the Asia and Pacific theatres during WWII (Gubler & Kuno 1997). More recently, several military operations have been affected by Dengue virus. Fourteen Dengue fever cases were confirmed from 90 U.S. military personnel hospitalised with febrile illness at the 86th Evacuation Army Hospital in Somalia during Operation Restore Hope from February to April 1993 (Kanesa-thasan *et al.* 1998). Dengue fever accounted for at least 30 % of the febrile illnesses among 406 hospitalised U.S. military personnel serving in Haiti in 1994 during Operation Uphold Democracy (Trofa *et al.* 1997). For the period from September 1999 to March 2000, 160 Dengue cases were clinically diagnosed in Australian military personnel at the 1st Forward Surgical Troop (1 FST) in East Timor during Operation Stabilise/Warden (Kitchener *et al.* 2001).

The clinical diagnosis of Dengue fever can be difficult. Acute symptoms may be misdiagnosed as the onset of hepatitis A, leptospirosis, malaria or another viral, bacterial or protozoan infection. Current antibody based ELISAs cannot provide a definitive result before 5 to 6 days after the onset of symptoms, and cell culture combined with indirect immunofluorescence assays requires approximately the same time. Both diagnostic methods require complex laboratory equipment. Subsequently, in the context of military operations, the diagnosis of viral infections in ADF personnel may necessitate shipment of specimens from remote areas to diagnostic laboratories, increasing costs and further delaying results.

PCR offers an alternative to antibody-based assays for virus detection. The method allows the specific detection of DNA and RNA sequences that are characteristic of any given cell or virus. PCR enzymatically amplifies a gene or gene portion to a detectable amount of PCR product (amplicon). Conventional PCR requires approximately 2 hours and is typically performed in reaction volumes of 50 to 100 µl. Upon completion of the reaction, an aliquot is subjected to gel electrophoresis to enable identification of a positive reaction. Detection of Alphaviruses and Flaviviruses via PCR requires an additional amplification step. The genetic information of these viruses is present as RNA that cannot be amplified directly. RNA has to be transcribed into a DNA copy (cDNA) by reverse transcription (RT) prior to amplification. A variety of RT-PCR kits are available that allow reverse transcription followed by amplification with the same reaction mix. However, reverse transcription adds approximately 30 minutes to the procedure. Furthermore, many PCR protocols for detection of viruses employ nested PCR or semi-nested PCR for rapid identification to increase the sensitivity of the test. A schematic presentation of nested PCR is shown in figure 1.

Published PCR protocols for detection of Alphaviruses and Flaviviruses were modified and evaluated for use with the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™), using either SYBR Green I or fluorescently labelled TaqMan® probes for detection. SYBR Green I detects the accumulation of PCR products that can include non-specific products and primer-dimers. TaqMan® probes allow detection of specific sequences and are less likely to yield false positive results. Principles of SYBR Green I- and TaqMan® probe- based detection are shown in figures 2 and 3. Selected protocols were optimised and tested over two months during a trial in East Timor as diagnostic tool for virus infections in ADF personnel. Specimens were screened with generic tests to determine the presence of any Alphavirus or Flavivirus.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Preparation of viral RNA and polymerase chain reaction

The High Pure Viral RNA extraction kit, Titan One Tube RT-PCR System, LightCycler–DNA Master FastStart SYBR Green I and LightCycler-DNA Master FastStart Hybridization Probes were purchased from Roche Diagnostics Australia Pty. Ltd. Amplification primers were produced by GeneWorks, Thebarton, South Australia. TaqMan® hydrolysis probes were manufactured by Genset Pacific, Lismore, New South Wales, Australia.

### 2.1.2 R.A.P.I.D.™ thermal cycler

The R.A.P.I.D.™ thermal cycler is a product of Idaho Technology Inc., Salt Lake City, Utah, US. LightCycler capillaries and LightCycler centrifuge adapters were purchased from Roche Diagnostics.

## 2.2 Methods

### 2.2.1 Extraction of viral RNA

Dengue virus serotypes 1, 2, 3 and 4 (Flavivirus) and Ross River virus (Alphavirus) were grown in C6/36 cells of *Aedes albopticus* essentially as described by Igarashi (1978). Cell culture supernatant was kindly provided by Dr. John Aaskov, Queensland University of Technology, Collaborative Centre for Arbovirus Reference and Research, Brisbane. Viral RNA was isolated with the High Pure Viral RNA Kit (Roche Diagnostics). Serum, plasma or cell culture supernatant (200 µl) was processed according to the manufacturer's instructions.

### 2.2.2 Published RT-PCR protocols

Published protocols (see table 1) for conventional RT-PCR (Pfeffer *et al.* 1997; Sánchez-Seco *et al.* 2001; Scaramozzino *et al.* 2002) were modified and evaluated for the R.A.P.I.D.™ using SYBR Green I as reporter fluorochrome. Modification and

optimisation of conventional PCR protocols for real-time PCR generally requires increase of the $MgCl_2$-concentration, decreasing the incubation periods for denaturation, primer annealing and extension and determination of the maximum number of amplification cycles that can be applied without causing significant primer-dimer formation. The protocol for Dengue virus detection and serotyping (Houng *et al.* 2001) utilises fluorogenic detection with TaqMan® probes. It was modified for the R.A.P.I.D.™ by using only the amplification primers and SYBR Green I detection before identification of Dengue serotypes with TaqMan® probes was attempted. All PCR amplifications included negative controls. Several of the amplification primers used in this study contain degenerate positions. These are specified by the internationally recognised mixed base code which is shown in table 2.

*Table 1. Published RT-PCR protocols modified for the R.A.P.I.D.™*

| **Assay Type** | **Source** |
|---|---|
| Semi-nested RT-PCR for genus-specific detection of Alphaviruses | Pfeffer *et al.* 1997 |
| Nested RT-PCR for genus-specific detection of Alphaviruses | Sánchez-Seco *et al.* 2001 |
| Semi-nested RT-PCR for genus-specific detection of Flaviviruses | Scaramozzino *et al.* 2001 |
| RT-PCR for identification of Dengue Virus serotypes 1-4 | Houng *et al.* 2001 |

*Table 2. Mixed base code for designation of degenerate primer positions**

| | | | |
|---|---|---|---|
| **R** | Adenosine, Guanosine | **Y** | Cytidine, Thymidine |
| **M** | Adenosine, Cytidine | **K** | Guanosine, Thymidine |
| **S** | Guanosine, Cytidine | **W** | Adenosine, Thymidine |
| **H** | Adenosine, Cytidine, Thymidine | **B** | Guanosine, Cytidine, Thymidine |
| **V** | Adenosine, Guanosine, Cytidine | **D** | Adenosine, Guanosine, Thymidine |
| **N** | Any Base | | |

*The letter I, occurring in several of the primer sequences, designates the base analogue Inosine, not a degeneracy.

#### 2.2.2.1 *Semi-nested RT-PCR for detection of Alphaviruses*

The assay utilises degenerate amplification primers targeting a conserved region of the non-structural protein 1 gene of the Alphaviruses. Primer designations, sequences and target positions are listed in table 3. Primer pair cM3W/M2W is used for RT-PCR and primer pair cM3W/M2W2 for the following semi-nested PCR.

*Table 3. Amplification primers for semi-nested Alphavirus RT-PCR (Pfeffer et al. 1997)*

| Primer | Sequence | Position |
|---|---|---|
| cM3W | ACATRAANKGNGTNGTRTCRAANCCDAYCC | Reverse, 568-597 |
| M2W | YAGAGCDTTTTCGCAYSTRGCHW | Forward, 164-186 |
| M2W2 | TGYCCNVTGMDNWSYVCNGARGAYCC | Forward, 288-313 |

#### 2.2.2.2 *Nested RT-PCR for detection of Alphaviruses*

The assay targets part of the non-structural protein 4 gene and utilises degenerate amplification primers that were designed based on highly conserved motifs in the nsP4 gene. They cover a consensus sequence in all Alphavirus sequences published to date. Primer designations, sequences and target positions are listed in table 4. Primer pair alpha1+/alpha1- is used for RT-PCR and primer pair alpha2+/alpha2- for the following nested PCR.

*Table 4. Amplification primers for nested Alphavirus RT-PCR (Sànchez-Seco et al. 2001)*

| Primer | Sequence | Position |
|---|---|---|
| alpha1+ | GAYGCITAYYTIGAYATGGTIGAIGG | forward, 6137-6172 |
| alpha1- | KYTCYTCIGTRTGYTTIGTICCIGG | reverse, 6509-6485 |
| alpha2+ | GIAAYTGYAAYGTIACICARATG | forward, 6315-6339 |
| alpha2- | GCRAAIARIGCIGCIGCYTYIGGICC | reverse, 6618-6593 |

#### 2.2.2.3 *Semi-nested PCR for detection of Flaviviruses*

The semi-nested RT-PCR is one of several Flavivirus detection assays that was extensively evaluated by Scaramozzino and co-workers. It was the only assay that enabled detection of all Flavivirus isolates tested. Scaramozzino *et al.* (2001) successfully transformed the RT-PCR into a semi-nested RT-PCR that showed significantly enhanced sensitivity. Primers target the non-structural region 5 ($NS_5$) of the Flavivirus genome. Primer designations, sequences and target positions are shown in table 5. Primer pair cFD2/MAMD is used for RT-PCR and primer pair cFD2/FS778 for the following semi-nested PCR.

*Table 5. Amplification primers for semi-nested Flavivirus RT-PCR (Scaramozzino et al. 2001)*

| Primer | Sequence | Position |
|---|---|---|
| cFD2 | GTGTCCCAGCCGGCGGTGTCATCAGC | reverse, 9232-9258 |
| MAMD | AACATGATGGGRAARAGRGARAA | forward, 9006-9029 |
| FS778 | AARGGHAGYMCDGCHATHTGGT | forward, 9044-9066 |

### *2.2.2.4 RT-PCR for identification of Dengue virus serotypes 1-4*

The assay targets the non-coding 3'-region of the dengue virus genome. Combinations of 4 forward primers, 2 reverse primers and 2 fluorescently labelled TaqMan® hydrolysis probes are applied to detect dengue virus and discriminate serotypes 1 - 4. Designations, sequences and target positions of the amplification primers and the two TaqMan® hydrolysis probes, DV.P1 and DV.P2, are shown in table 6. Primer/probe combinations are DV1.U/DV.P1/DV.L1 for serotype 1, DV2.U/DV.P1/DV.L1 for serotype 2, DV3.U/DV.P2/DV.L1 for serotype 3 and DV4.U/DV.P2/DV.L2 for serotype 4.

*Table 6. Primers/probes for Dengue virus serotype identification (Houng et al. 2001)*

| Primer/Probe | Sequence | Position |
|---|---|---|
| DV1.U | ACACCAGGGGAAGCTGTATCCTGG | forward, 236-261 |
| DV2.U | AAGGTGAGATGAAGCTGTAGTCTC | forward, 236-261 |
| DV3.U | AGCACTGAGGGAAGCTGTACCTCC | forward, 236-261 |
| DV4.U | AAGCCAGGAGGAAGCTGTACTCCT | forward, 236-261 |
| DV.P1 | CTGTCTCCTCAGCATCATTCCAGGCA | TaqMan®, 341-366 |
| DV.P2 | CTGTCTCTGCAACATCAATCCAGGCA | TaqMan®, 341-366 |
| DV.L1 | CATTCCATTTTCTGGCGTTCT | reverse, 368-388 |
| DV.L2 | CAATCCATCTTGCGGCGCTCT | reverse, 368-388 |

### 2.2.3 Modification of protocols for the R.A.P.I.D.™

#### *2.2.3.1 Reverse transcription*

The Titan One Tube RT-PCR System (Roche Diagnostics) was employed for transcription of viral RNA into cDNA. Reaction volumes were reduced to 25 µl (the manufacturer recommends 50 µl), which is the maximum volume that can be accommodated by the R.A.P.I.D.™ capillaries. Reaction mixtures contained 0.4 µM reverse primer, 5 µl RNA extract, 3 mM $MgCl_2$, 5 mM dithiothreitol, 200 µM dNTPs (final concentrations) and 0.5 µl reverse transcriptase and Expand High Fidelity PCR System enzyme mix (the manufacturer does not specify units per µl). Reactions were incubated at 50°C for 30 min and analysed on ethidium bromide stained 2 % agarose gels.

#### *2.2.3.2 RT-PCR*

Reaction mixtures were as for reverse transcription with the addition of 0.4 µM forward primer. Following reverse transcription the reactions were denatured at 95°C for 2 min and amplified over 30 cycles of denaturation at 95°C for 5 sec, primer annealing at 52°C for 10 sec and extension at 72°C for 10 sec. Completed reactions were analysed as above.

#### *2.2.3.3 Nested and semi-nested PCR*

The LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) was used for nested/semi-nested PCR. A total reaction volume of 20 µl contained 0.2 – 1.0 µM forward and reverse primer, 1 µl of a 1:10 dilution – 5 µl of undiluted completed RT-PCR reaction, 2 – 5 mM $MgCl_2$ and 2 µl of 10x reaction buffer. The reaction buffer provided by the supplier contains $MgCl_2$ and dNTPs. The final $MgCl_2$ concentration in the reaction mix resulting from the 10x buffer is 1 mM and can be increased with additional $MgCl_2$ as required. The manufacturer does not disclose the concentration of dNTPs in the 10x buffer. Optimised SYBR Green I amplification protocols are specified under 'results and discussion'. Completed reactions were analysed by generating a melting curve of the amplification products to enable discrimination of positive reactions and accumulation of primer-dimers. The R.A.P.I.D.™ incubation profile for melting curves was 94°C for 0 sec (instrument setting; means go to next target temperature once set temperature is reached), 50°C for 45 sec and heating to 95°C at a temperature transition rate of 0.1°C/sec.

#### *2.2.3.4 TaqMan® probe PCR*

The LightCycler-FastStart – DNA Master Hybridization Probes kit (Roche Diagnostics) was used for TaqMan® Probe PCR. A total reaction volume of 20 µl contained 0.5 – 1.0 µM forward and reverse primer, 0.02 – 0.2 µM TaqMan® probe, 1 µl of a 1:10 dilution – 5 µl undiluted reverse transcription reaction, 2 – 5 mM $MgCl_2$ and 2 µl 10x reaction buffer. The $MgCl_2$ resulting from the 10x buffer is 1 mM. The dNTP concentration of the buffer is not specified. PCR amplification was carried out for 50 cycles with different incubation temperatures and incubation times.

### 2.2.4 Field trial of the R.A.P.I.D.™

The R.A.P.I.D.™ was deployed from the Australian Army Malaria Institute (AAMI) to the United Nations Military Hospital, East Timor, under operational command of the 2nd Battalion, Royal Australian Regiment, from November 17, 2001 – January 25, 2002. The purpose of the deployment was to evaluate the R.A.P.I.D.™ with adapted RT-PCR assays for the diagnosis of dengue virus in East Timor. Ethical approval for the trial was obtained from the Australian Defence Human Research Ethics Committee (ADHREC), approval number 277/01.

Under the protocols adopted for the trial, each blood sample was screened with universal primer sets for Flavivirus and Alphavirus infections. Samples that tested positive for Flavivirus were subsequently tested for Dengue Virus serotypes 1 – 4. All PCRs included positive and negative controls.

#### *2.2.4.1 R.A.P.I.D.™ Screening of asymptomatic personnel*

Screening of healthy, asymptomatic personnel was carried out at Marko and the company headquarters in Aidabaleten on the 2nd and 9th of December 2001 to determine whether the R.A.P.I.D.™ could potentially produce false positive results. Screening of these personnel was performed after obtaining informed consent from personnel enrolled in the mefloquine anti-malarial drug trial (ADHREC approval number 249/01). The serum samples obtained for mefloquine pharmokinetic analysis were tested for Alphavirus and Flavivirus RNA with the RT-PCR assays established for the R.A.P.I.D.™. Cell culture isolation and indirect immunofluorescence assays were used to confirm the absence of Alphavirus and Flavivirus infection independent of the R.A.P.I.D.™ in the laboratories of AAMI.

#### *2.2.4.2 R.A.P.I.D.™ Screening of clinical cases*

Specimens from 32 patients presenting at the UN Military hospital were examined as described under 2.2.4.

# 3. Results and Discussion

## 3.1 Extraction of viral RNA

Yields of viral RNA obtained with the High Pure Viral RNA kit from 200 µl cell culture supernatant, blood or sera were sufficient for the generation of cDNA during the initial reverse transcriptase reaction. Prolonged storage of extracted RNA was problematic. In several cases, RNA that was successfully transcribed into cDNA immediately after extraction failed to provide cDNA after storage at -20°C for as short as 24 h. To avoid degradation of viral RNA it is necessary to treat all non-commercial storage and dilution buffers that are made in the laboratory with diethyl pyrocarbonate (DEPC), a specific ribonuclease inhibitor.

## 3.2 Adapted R.A.P.I.D.™ protocols

### 3.2.1 Detection of Alphaviruses (Pfeffer *et al.* 1997)

The protocol could not be adapted for the R.A.P.I.D.™. No bands were observed in the analysis of RT-PCR solutions on ethidium bromide stained 2 % agarose gels. It was assumed that the amount of amplification product was too low for detection via agarose gel electrophoresis, and different volumes, ranging from 1 µl of a 1:10 dilution to 5 µl of the RT-PCR, were used as template for the semi-nested PCR. In all cases, semi-nested PCR showed a positive result as indicated by the fluorescence profile. However, analysis by melting curve revealed that the positive fluorescence profile was due to primer-dimer formation, and no specific amplification product had been generated. Increasing the stringency of the semi-nested PCR by decreasing the $MgCl_2$ concentration and increasing the primer annealing temperature up to 5°C above the melting points ($T_m$) of the amplification primers did not generate specific amplification product. It was concluded that the high degree of degeneracy of the amplification primers with up to 13 positions with multiple degeneracies per primer rendered the assay unsuitable for R.A.P.I.D.™ application.

### 3.2.2 Detection of Alphaviruses (Sánchez-Seco *et al.* 2001)

Analysis of RT-PCR aliquots on agarose gels failed to detect a product but nested PCR yielded a positive result, and the melting curve showed the presence of a specific amplification product and no significant primer-dimer formation. Agarose gel electrophoresis confirmed that the amplification product had the expected size of 195 bp (data not shown).

The amount of RT-PCR product added to the nested PCR had to be limited to 1 µl of a 1:10 dilution. Larger or undiluted aliquots caused significant inhibition of the nested PCR, suggesting that the reaction buffer of the Titan RT-PCR kit is incompatible with the polymerase of the FastStart – DNA Master SYBR Green I kit (data not shown). The optimised thermal profile comprised denaturation at 95°C for 10 min and amplification over 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 52°C for 20 sec and extension at 72°C for 10 sec. Amplification primers were titrated to 0.4 µM each and $MgCl_2$ to 3 mM. Under the above conditions a positive reaction was completed after 40 cycles. No primer-dimer formation was detected in the positive reaction and the negative control. Fluorescence profiles and melting curves are shown in figures 5 and 6.

### 3.2.3 Detection of Flaviviruses

Similar to the nested PCR for detection of Alphaviruses, no RT-PCR product was detected by agarose gel electrophoresis, but the semi-nested PCR with 1 µl of 1:10 diluted RT-PCR showed a positive result. The melting curve confirmed the presence of a specific amplification product. The correct size of the product (200 bp) was determined as above (data not shown).

The optimised thermal profile was denaturation at 95°C for 10 min and amplification over 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 54°C for 5 sec and extension at 72°C for 10 sec. Amplification primers were titrated to 0.7 µM each and

$MgCl_2$ to 3 mM. A positive reaction was completed after approximately 35 cycles. At that stage, no primer-dimer formation was observed in the positive reaction and the negative control. Fluorescence profiles and melting curves are shown in figures 7 and 8.

### 3.2.4 Detection of Dengue virus serotype 1 - 4

Modification and optimisation of the published PCR assay for detection and discrimination of Dengue virus serotypes was initially attempted with the LightCycler - FastStart DNA Master SYBR Green I without employing the fluorogenic probes DV.P1 and DV.P2. Excessive primer-dimer formation occurred in all reactions containing viral cDNA and in the negative control reactions. No specific amplification product could be detected by melting curve and agarose gel electrophoresis. The problem could not be rectified by increasing the stringency of the reactions through increased annealing temperature or decreased $MgCl_2$ and primer concentration. When tested in the generic Flavivirus assay the RNAs of all four Dengue virus serotypes showed positive results, indicating that the lack of specific amplification product was not due to degraded viral RNA.

Serotyping with the LightCycler - FastStart DNA Master Hybridisation Probes with inclusion of the fluorogenic probes DV.P1 and DV.P2 was also unsuccessful. No increase in fluorescence above background was observed during the amplification and no product formation was detected by agarose gel electrophoresis. The reason for failure of the method could not be established, and the attempt to adapt the serotyping protocol for the R.A.P.I.D.™ was abandoned.

## 3.3 Field trial of the R.A.P.I.D.™

### 3.3.1 R.A.P.I.D.™ screening of asymptomatic personnel

None of the 87 individuals tested was found to be subclinically viraemic for Alphaviruses or Flaviviruses at the time of testing. The result suggested that the generic RT-PCR assays are unlikely to generate false positive reactions from clinical specimens.

### 3.3.2 R.A.P.I.D.™ screening of clinical cases

#### *3.3.2.1 Generic tests for Alphaviruses and Flaviviruses*

No Alphavirus was detected in any of the clinical patients. Of the 13 subjects confirmed viraemic by the Collaborative Centre for Arbovirus Reference and Research, 7 patients tested positive for Flavivirus RNA. A subject was considered positive for Flavivirus RNA if the $T_m$ of the PCR product was within half a standard deviation of the positive control $T_m$. Six patients tested false negative with the modified SYBR Green I PCR protocol of Scaramozzino *et al.* (2001). Problems with centrifugation during RNA isolation may have contributed to these false negative results. The Eppendorf centrifuge used for RNA isolation malfunctioned under field conditions and was replaced with a mini centrifuge C-1200 supplied with the R.A.P.I.D.™. This centrifuge is likely to lack sufficient G force for the High Pure Viral RNA kit used for RNA extraction. The system is based on silica gel spin column extraction of viral RNA from human blood, with high

G forces required for purification of RNA. Five of the six negative results were recorded after the Eppendorf centrifuge failure.

#### 3.3.2.2 *Dengue virus serotyping*

Dengue virus was serotyped for all 13 subjects found viraemic for Dengue by serotype specific indirect immunofluorescent assay following cell culture isolation. Further, isolates were serotyped at the WHO Collaborating Centre for Arbovirus Reference and Research using a new multiplex RT-PCR system (not available at the time the trial was conducted) capable of diagnosing Dengue virus, Japanese Encephalitis virus and Yellow Fever virus. As shown in table 7, Dengue serotypes 1 and 4 were found to be predominant.

Subject 277/01-079 was found to have a Dengue virus serotype 2 infection concurrent with *Plasmodium vivax* malaria. A history was taken from all patients at the time of consent for aspirin and paracetamol (acetaminophen) use in the previous 48 hours as both are known to be toxic for cell cultures. The serum from 10 patients was noted to be cytotoxic for the C6/36 mosquito cells used to isolate mosquito borne viruses during the trial. If these cell cultures die during the 7-day incubation period, a false negative result can occur. For the majority of patients where cell death did occur, the cause was not determined. However, patient 277/01-079 was diagnosed with *Plasmodium vivax* malaria infection, and blood samples were obtained on the 5th, 9th, 11th and 12th of December 2001. Of the four blood samples collected from this patient during the febrile phase of illness, samples from the 5th – 9th of December 2001 could not be tested for Flavivirus infection using traditional cell culture methods. Medications administered to this patient included paracetamol, metoclopramide, endensetron, fansidar and chloroquine. Samples collected on the 11th – 12th December were sufficiently non-cytotoxic to allow isolation of Dengue virus serotype 2. However, both the multiplex RT-PCR from the WHO Collaborative Centre for Arbovirus Reference and Research and R.A.P.I.D.™ results indicated Dengue/Flavivirus infection on 5th December 2001. This adds to the utility of the R.A.P.I.D.™ in the field when drug treatments are likely to interfere with traditional diagnostic techniques.

# 4. Conclusions

The trial in East Timor demonstrated that the R.A.P.I.D.™ has the potential of a diagnostic tool for real-time detection/diagnosis of microbial pathogens. Although it did not achieve its desired aim of detecting and identifying Dengue virus serotypes the trial showed that conventional PCR methods for the detection of Alphaviruses and Flaviviruses could be modified for use in the R.A.P.I.D.™. False negative results could be traced to equipment failure and can be avoided by including adequate backup equipment in future deployments. Further trials need to be conducted to develop real-time, in the field assays for the diagnosis of infectious diseases likely to afflict ADF personnel.

Shortcomings of the PCR assays in identifying Dengue virus serotypes pose a more complex problem. The fact that all four Dengue virus serotypes were not detected with the amplification primers published by Houng and co-workers (2001) might indicate

that the target sequences of the viruses used in this study are different and do not match the published primers, particularly considering that these viruses could be detected with amplification primers for generic detection of Flaviviruses as published by Scaramozzino and co-workers (2001). Several researchers have voiced concern that the target sequences in the isolates investigated by Houng *et al.* (2001) might differ in isolates from various geographic distributions (Reid, personal communication). Furthermore, mutations of the target gene in different Dengue virus strains could compound the problem of serotype identification. Sequence analysis of Dengue viruses from different origins is currently being undertaken and may enable the design of serotyping primers that are universally applicable.

The R.A.P.I.D.™ is a diagnostic tool that is easily deployable as it showed to be very robust and requires comparatively few accessories to enable diagnostic testing in the field. It is suitable for detection of a wide range of infectious diseases likely to affect ADF personnel. Apart from vector transmitted viral infections, melioidosis, tuberculosis and malaria are among the prime targets for R.A.P.I.D.™ based diagnostics.

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*Table 7. Summary of clinical results from the East Timor trial*

| Study no. | Date of Onset | Sample Collection date | Days post onset | Diagnosis | Serology Result | Seroconversion date | RAPID Screening | RAPID Serotyping | WHO Reference Lab Result |
|---|---|---|---|---|---|---|---|---|---|
| 277/01-078 | 01-Dec-01 | 11-Dec-01 | 10 | presumptive TB | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-079 | 05-Dec-01 | 05-11 Dec 01 | 1 | vivax malaria & dengue fever | IgM/IgG DETECTED | 12-Dec-01 | Detected | NOT DETECTED | Dengue 2 isolated 11 Dec 01 |
| 277/01-080 | 15-Dec-01 | 16-Dec-01 | 1 | Dengue fever | NOT AB TESTED | Not Done | Detected | NOT DETECTED | Dengue 2 isolated |
| 277/01-081 | 14-Dec-01 | 17-Dec-01 | 3 | Non specific viral illness | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-082 | 15-Dec-01 | 18-Dec-01 | 3 | Pyrexia of unknown origin | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-083 | 17-Dec-01 | 18-Dec-01 | 1 | Dengue fever | IgM DETECTED | 24-Dec-01 | Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-084 | 17-Dec-01 | 24-Dec-01 | 3 | Pharyngitis | NOT DETECTED | 20-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-085 | 19-Dec-01 | 20-Dec-01 | 1 | Dengue fever | IgM/IgG DETECTED | 26-Dec-01 | Detected | NOT DETECTED | Dengue 1 isolated |
| 277/01-086 | 13-Dec-01 | 20-Dec-01 | 7 | Non specific viral illness | NOT DETECTED | 20-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-087 | 14-Dec-01 | 21-Dec-01 | 7 | Upper Respiratory Tract Infection | NOT DETECTED | 21-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-089 | 20-Dec-01 | 22-Dec-01 | 2 | Dengue fever (2nd episode) | IgG DETECTED | 26-Dec-01 | Not Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-090 | 21-Dec-01 | 22-Dec-01 | 1 | Ascaris lumbricoides infection | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-091 | 19-Dec-01 | 22-Dec-01 | 3 | Pyrexia of unknown origin | NOT DETECTED | 31-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-092 | 17-Dec-01 | 22-Dec-01 | 5 | vivax malaria | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-093 | 20-Dec-01 | 22-Dec-01 | 2 | Dengue fever | IgM/IgG DETECTED | 28-Dec-01 | Detected | NOT DETECTED | Dengue 1 isolated |
| 277/01-094 | 24-Dec-01 | 26-Dec-01 | 2 | Bronchitis & pharyngitis | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-095 | 25-Dec-01 | 26-Dec-01 | 1 | Dengue fever | NOT AB TESTED | Not Done | Not Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-096 | 25-Dec-01 | 26-Dec-01 | 1 | Non specific viral illness | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-097 | 20-Dec-01 | 26-Dec-01 | 7 | Pyrexia of unknown origin | NOT DETECTED | 28-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-098 | 24-Dec-01 | 26-Dec-01 | 2 | Pyrexia of unknown origin | NOT DETECTED | 07-Jan-02 | Not Detected | Not Done | Not Isolated |
| 277/01-099 | 26-Dec-01 | 26-Dec-01 | 1 | Dengue fever | IgM/IgG DETECTED | 31-Dec-01 | Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-100 | 26-Dec-01 | 28-Dec-01 | 2 | Non specific viral illness | NOT DETECTED | 31-Dec-01 | Not Detected | Not Done | Not Isolated |
| 277/01-101 | 24-Dec-01 | 27-Dec-01 | 3 | Pyrexia of unknown origin | NOT DETECTED | 03-Jan-02 | Not Detected | Not Done | Not Isolated |
| 277/01-102 | 26-Dec-01 | 29-Dec-01 | 3 | Pyrexia of unknown origin | NOT DETECTED | 08-Jan-02 | Not Detected | Not Done | Not Isolated |
| 277/01-103 | 25-Dec-01 | 28-Dec-01 | 3 | Dengue fever | IgM DETECTED | 28-Dec-01 | Not Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-104 | 28-Dec-01 | 01-Jan-02 | 5 | Dengue fever | IgM DETECTED | 03-Jan-02 | Not Detected | NOT DETECTED | Dengue 1 isolated |
| | | | | | | | | | |
| 277/01-106 | 31-Dec-01 | 03-Jan-02 | 4 | Dengue fever | IgM DETECTED | 05-Jan-02 | Not Detected | NOT DETECTED | Dengue 1 isolated |
| | | | | | | | | | |
| 277/01-108 | 03-Jan-01 | 05-Jan-02 | 2 | Dengue fever | NOT DETECTED | 15-Jan-02 | Not Detected | NOT DETECTED | Dengue 4 isolated |
| 277/01-109 | 06-Jan-01 | 07-Jan-02 | 1 | Upper Respiratory Tract Infection | NOT AB TESTED | Not Done | Not Detected | Not Done | Not Isolated |
| 277/01-110 | 05-Jan-01 | 04-Jan-02 | 2 | Dengue fever | IgM DETECTED | 12-Jan-02 | Detected | NOT DETECTED | Dengue 1 isolated |

**White rows:** Serology negative for Dengue
**Yellow and pink rows:** Serology positive for Dengue

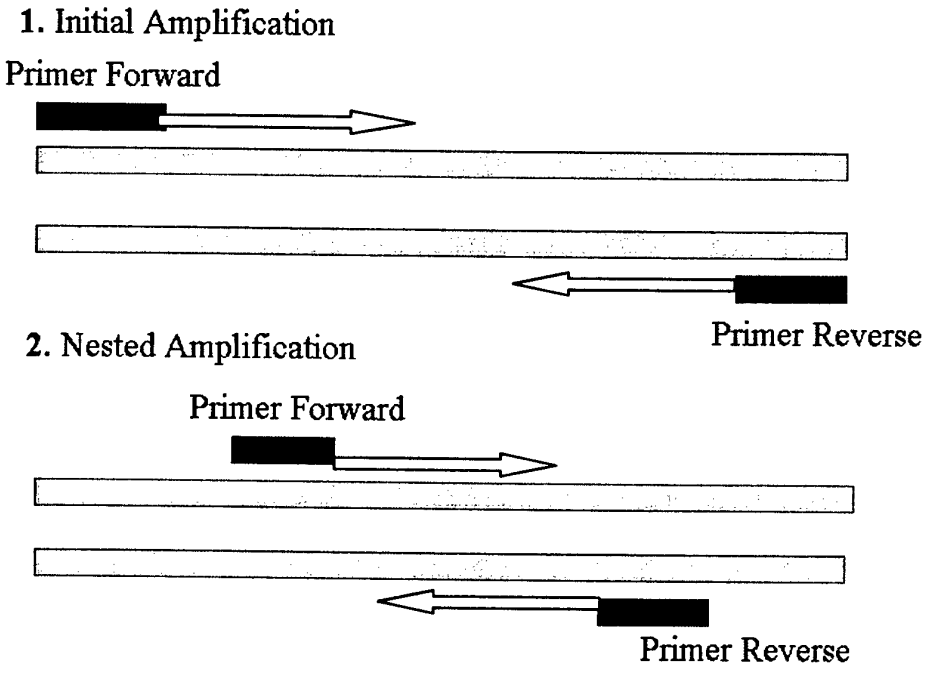


*Figure 1. Nested PCR employs one set of primers during initial amplification. A second 'nested' amplification is then carried out with primers targeting regions within the initial amplicon, resulting in a shorter amplification product. Arrows indicate the direction of primer extension.*

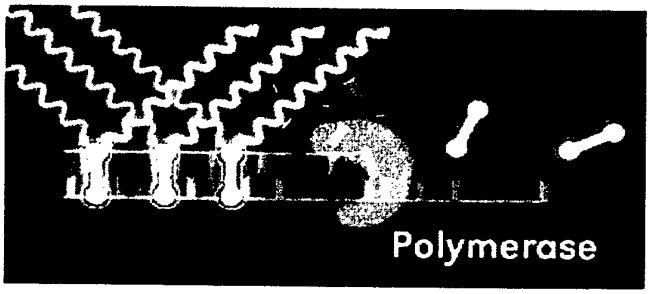


*Figure 2. SYBR Green I is incorporated into the double stranded DNA generated by the DNA polymerase. It emits a fluorescent signal when irradiated with excitation light. (Image from Idaho Tech.)*

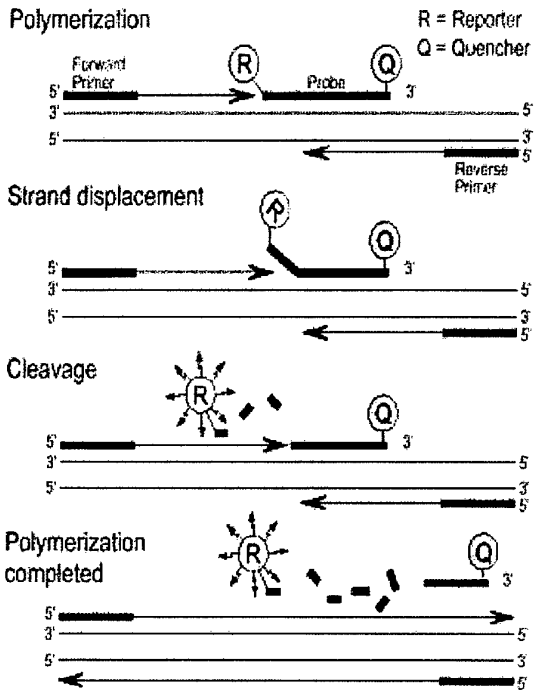


*Figure 3. TaqMan® probes are cleaved by DNA polymerase. Reporter fluorochrome and quencher are separated and a fluorescent signal is emitted. (Image from Idaho Tech.)*

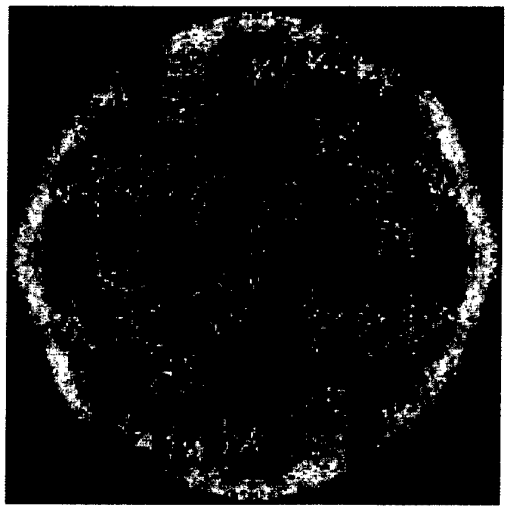

*Figure 4. The protein E (envelope protein, colour coded blue, green and red) forms the Dengue virus capsid containing positive strand RNA. During preparation of viral RNA the capsid is destroyed and RNA retained. (Image from ABC Science News website)*

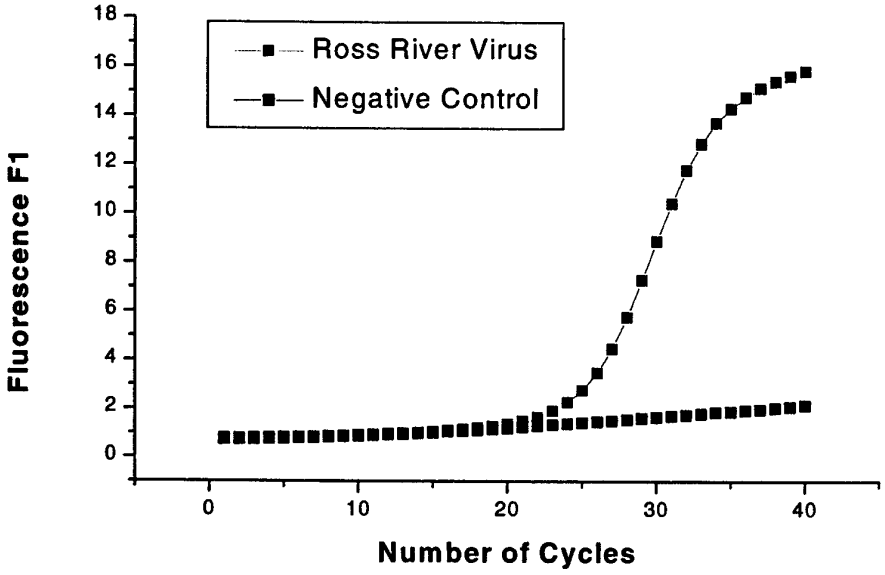


*Figure 5. Fluorescence amplification profile of Ross River virus and a negative control.*

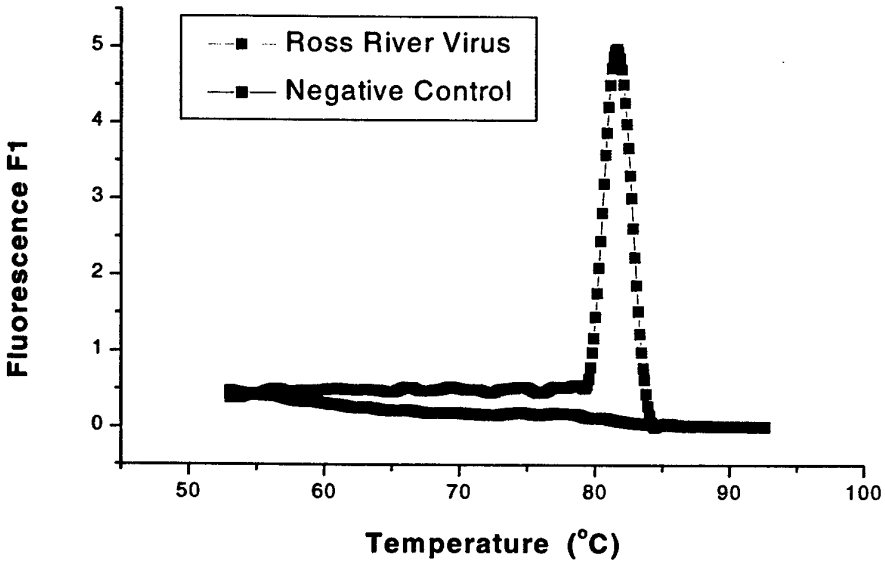


*Figure 6. The melting curve shows absence of primer-dimer formation in both positive reaction and negative control.*

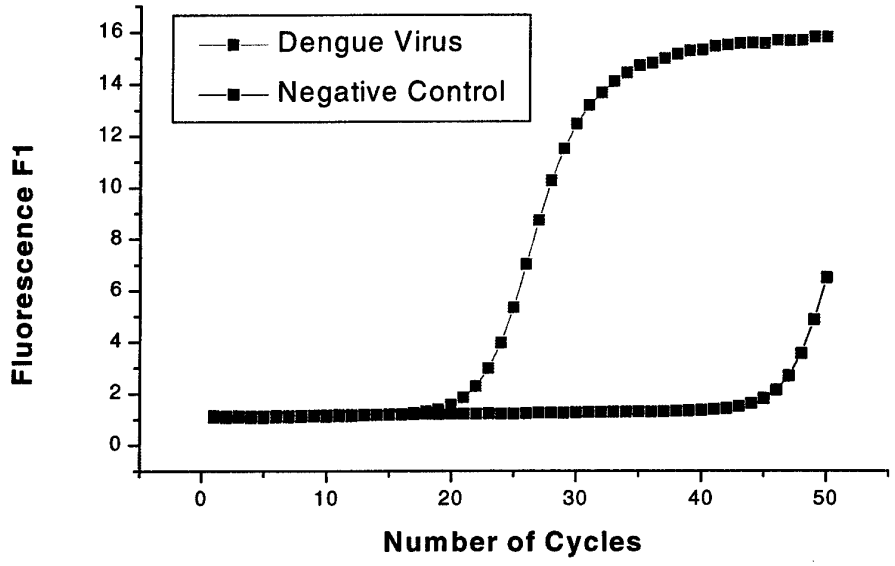


*Figure 7. Fluorescence amplification profile of Dengue virus and negative control. The negative control shows primer-dimer formation after 40 cycles.*

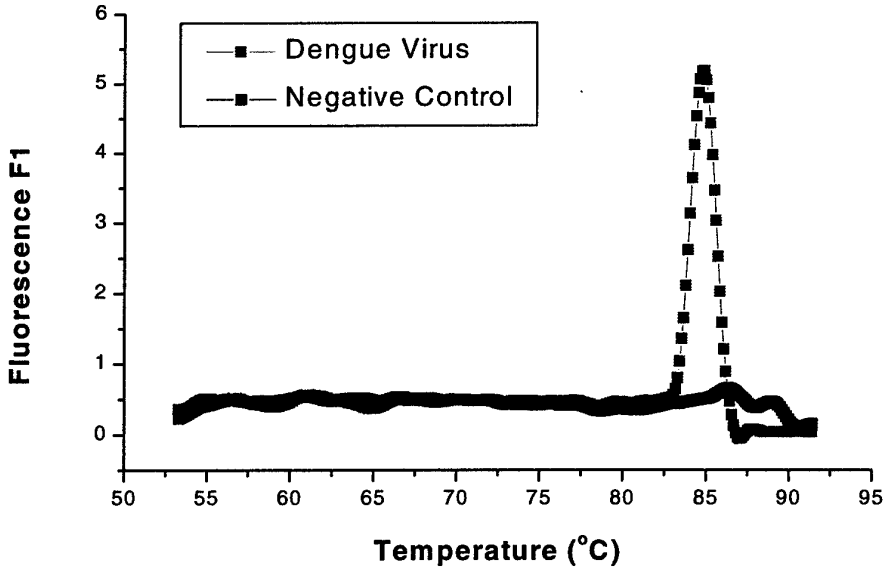


*Figure 8. The melting curve shows specific product formation in the positive reaction and a low degree of primer-dimer formation in the negative control.*

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19. ABSTRACT

Polymerase chain reaction (PCR) assays for detection of vector transmitted virus infections in humans designed for conventional PCR thermal cyclers were modified for real-time PCR using a LightCycler. Two reporter fluorochromes, the double stranded DNA specific dye SYBR Green I, and 6-hydroxy fluorescein conjugated to TaqMan® DNA probes, were employed to detect positive reactions. Following optimisation of the assays for real-time PCR, portable real-time PCR equipment, the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.™), was deployed to East Timor and a field trial was conducted from November 2001 until January 2002. Blood samples from a total of 143 army personnel were screened with the R.A.P.I.D.™ for virus infections. These included 33 samples from patients showing clinical symptoms. Results obtained with the R.A.P.I.D.™ were compared to results from serological testing and cell culture analysis.